Journal of Chemical Ecology, Vol. 21, No. 1, 1995

PRODUCTION OF PHEROMONE BY HAIRPENCIL GLANDS OF MALES OBTAINED FROM INTERSPECIFIC HYBRIDIZATION BETWEEN *Heliothis virescens* AND *H. subflexa* (LEPIDOPTERA: NOCTUIDAE)

PETER E.A. TEAL,* and ANDREA OOSTENDORP

Insect Attractants Behavior and Basic Biology Research Laboratory, USDA PO Box 14565, Gainesville, Florida, 32604

(Received May 16, 1994; accepted September 29, 1994)

Abstract—Pheromone produced by the hairpencil glands of interspecific hybrid- and backcross-generation males from crosses between *Heliothis virescens* (F.) with *H. subflexa* (Gn.) was studied. Males of reciprocal F_1 hybrids, all of which had hairpencil glands morphologically similar to those of *H. virescens*, produced neither the same pheromone blend nor amounts of pheromone that were produced by males of *H. virescens*. Instead, these hybrid males produced pheromone that was quantitatively and qualitatively similar to that produced by *H. subflexa*. Hairpencil gland extracts from males obtained from backcrossing F_1 females of either cross to males of *H. subflexa* were the same as those of *H. subflexa*. However, extracts from backcross males of crosses between F_1 females and *H. virescens* were variable. Some extracts from these backcross males were like those of *H. virescens* while others were either like *H. subflexa* or were intermediate between those of the parent species. These results showed that the production of pheromone by the hairpencil glands of hybrid and backcross males is under the dominant regulation of autosomal genes of the *H. subflexa* genome.

Key Words—Hairpencils, sex pheromone, *Heliothis*, autosomes, sex chromosomes.

INTRODUCTION

Heliothis virescens (F.) and *H. subflexa* (Gn.) are closely related species of noctuid moths that can be induced to hybridize in no-choice laboratory condi-

*To whom correspondence should be addressed.

0098-0331/95/0100-0059$07.50/0

tions (Laster, 1972; Proshold and LaChance, 1974). However, the two species are reproductively isolated, and there is no evidence for interspecific hybridization in nature. Reproductive isolation between the two species is maintained by both pre- and postmating mechanisms. The principal means by which species integrity and reproductive isolation are maintained is through the use of highly specific blends of female produced pheromones (Tingle et al., 1978; Heath et al., 1991). These pheromones attract males from varying distances and are the first step in the mating ritual. However, the success of mating depends also upon female acceptance of courting males.

Among many moth species, acceptance of males by females is mediated chemically by pheromones released from male hairpencil glands during courtship (Birch and Hefetz, 1987). Males of *H. virescens* partially expose genital hairpencil glands just prior to landing near a female that is releasing pheromone and fully expose these structures just prior to copulation (Teal et al., 1981). Exposure of the hairpencils results in a sudden release of a blend of pheromone components in which hexadecan-1-ol and hexadecan-1-ol acetate are present in greatest amounts (Teal and Tumlinson, 1989). Studies have indicated that effective hairpencil exposure is important for reproductive success (Teal et al., 1981; Teal and Tumlinson, 1989). Similarly, males of *H. subflexa* expose the genital segments prior to attempting to copulate with conspecifics (Cibrian-Tovar and Mitchell, 1991).

Recent studies have shown that inheritance of the hairpencil glands by interspecific hybrid and backcross males is controlled by a dominant gene(s) on the male sex chromosome (Z chromosome) of *H. virescens* (Teal and Oostendorp, 1993). Thus, all hybrid and any backcross males that obtain one of the two Z chromosomes from *H. virescens* have the same type of hairpencil glands as males of *H. virescens* because males of Lepidoptera are homogametic and have two sex chromosomes (denoted ZZ). Given that the hairpencil pheromone is sex specific and that the structure of the hairpencil glands was regulated by the chromosomes, we were interested in knowing if the production of pheromone by these male glands was regulated by sex-linked inheritance or autosomal genes as reported for pheromone production by females of these hybrids (Klun et al. 1982). The following reports the results of studies in which the amounts and ratios of hairpencil pheromone present in extracts from hybrid and backcross males were compared to those present in extracts from males of the parent species.

METHODS AND MATERIALS

Insect Cultures. Both *H. virescens* and *H. subflexa* parent stocks were obtained as pupae from colonies maintained at our laboratory. The founding colonies of both species were begun from larvae collected in fields in Alachua

County, Florida, in 1986. Interspecific hybridizations were conducted by combining five males of one species with five females of the other species in 500-ml cardboard cartons with screen tops. Three cartons were set up each time a cross was conducted. Cartons were inspected daily for the presence of larvae. Approximately equal numbers of larvae from each of the three cartons were reared individually as described by Teal and Oostendorp (1993). All insects were held in environmental chambers having a reversed 14:10 (light–dark) photoperiod at 26°C and 55% relative humidity. Four separate crossing experiments, conducted at different times, were undertaken for each interspecific cross. Female hybrids from each rearing were backcrossed to males of the parent species in a similar manner. Thus, all experiments were replicated four times and hybrid and backcross insects from each crossing experiment were the progeny of at least three matings between different parents. After adult emergence, males were aged for two to four days prior to use. No males from hybrid or backcross lines could be used for crossing experiments because of sterility (Laster, 1972; Proshold and LaChance, 1974; Karpenko and Proshold, 1977).

Hairpencil Gland Excision and Extraction of Pheromone. Studies on the pheromone produced by males of hybrid and backcross insects were conducted using insects sampled during the fifth to sixth hours of the second to fourth scotophase after adult emergence. This time period corresponded to the peak periods of pheromone production by females of *H. virescens* and *H. subflexa* (Heath et al., 1991; Teal et al., 1993).

Tissue used to obtain extracts included the terminal abdominal segments, which are normally retracted into the abdomen. These segments, bearing the hairpencil glands, were exposed by application of pressure to the anterior abdominal segments. The exposed genital segments were then excised and placed into 60 μl of hexane containing 20 ng each of octadecane and nonadecane, internal standards used for quantitation and calculation of relative retention indices. After ca. 10 min, the solvent was removed and stored in a clean microvial at −60°C until analysis. Prior to chemical analysis, the volume of the extracts was reduced to ca. 5 μl under a stream of nitrogen.

Chemical and Data Analysis. Routine chemical analyses were conducted by capillary gas chromatography (GC) using Hewlett Packard 5890 GCs equipped with split/splitless injectors and flame ionization detectors. Capillary columns used for analysis included both apolar phase (SPB1) and polar phase (Supelcowax 10) columns. The fused silica columns were 30 m × 0.25 mm ID and had a film thickness of 0.25 μm. Conditions of chromatography were as follows: initial oven temperature 60°C, oven temperature increased after 2 min at 30°/min, final temperature 200°C. Helium was used as the carrier gas at a linear flow velocity of 18 cm/sec. Individual extracts were injected in the splitless mode with the injector being purged after 0.5 min. Data were acquired with a Nelson 3000 data acquisition system and analyzed by using Nelson 2600 soft-

ware. Quantitative data on the two principal components of the pheromone of *H. virescens*, hexadecan-1-ol (16:OH) and hexadecan-1-ol acetate (16:Ac), were transferred to Lotus 1-2-3 spreadsheets and ratios were calculated. Statistical analyses were performed on these data using NCSS statistical software. For the purposes of this study, we considered the ratio of components in extracts obtained from hybrid and backcross males to be like *H. virescens* if the ratio of 16:AC to 16:OH was 3.5:1 or greater or to be like *H. subflexa* if the ratio was 1:1 or less. Extracts containing more than a total of 125 ng of pheromone were considered to be like *H. virescens* and those containing less than 10 ng were considered to be like *H. subflexa*. All other values were considered to be intermediate between the two species.

Mass spectral analyses were used to confirm the identities of compounds present in extracts obtained from hairpencil glands of the parent species and in samples selected at random from hybrid and backcross insects. A Nermag R1010 MS operated in the chemical ionization mode (methane reagent gas) and interfaced to a Hewlett Packard 5790 GC was used. The SPB1 column used for GC-MS studies was operated as described above.

RESULTS AND DISCUSSION

Parent Species. Extracts of the hairpencil glands from individual males of *H. virescens* analyzed during the present study showed that 16:Ac and 16:OH were the principal components of the pheromone, confirming previous findings (Teal and Tumlinson, 1989). The mean amount of 16:Ac was 286.1 ng (SE = 24.84, N = 15, max = 430.5 ng, min = 128.4 ng) and for 16:OH was 70.4 ng (SE = 8.89, N = 15, max = 127.64 ng, min = 17.5 ng, mean ratio Ac:OH = 4:1). These values were also in agreement with those of earlier work (Teal and Tumlinson, 1989).

Analysis of extracts of the terminal abdominal segments of males of *H. subflexa* did not reveal the presence of any compounds that were unique to this species. Minute amounts of 16:OH (mean = 1.36 ng, SE = 0.36) were found in 18 of the 50 extracts analyzed by GC. Data supporting the presence of 16:OH was obtained from GC-MS analysis of an extract of 15 glands on the basis of retention time and selected ion monitoring using major ions determined from analysis of synthetic 16:OH (m/z 242 = M, 241 = M-1 (parent ion), 225 = (M+1)-18, 224 = M-18). Only six of the 50 samples contained detectable amounts of 16:Ac (mean = 0.39 ng, SE = 0.28) and the presence of the acetate could not be confirmed by GC-MS. The maximum amount of 16:AC plus 16:OH found in an individual extract was 7.3 ng (3.1 ng 16:Ac plus 4.2 ng 16:OH).

F_1 Hybrids. Extracts of the hairpencil glands from both of the hybrid lines

contained small amounts of pheromone. The mean amounts of 16:Ac and 16:OH for *H. subflexa* female × *H. virescens* male (S line) males were 0.8 ng (SE = 0.13, N = 125), and 3.17 ng (SE = 0.29, N = 125) and for *H. virescens* female × *H. subflexa* male (V line) males were 1.01 ng (SE = 0.20, N = 102) and 3.76 ng (SE = 0.35, N = 102), respectively (Figure 1). Neither the mean total amounts of 16:OH plus 16:Ac nor the mean amounts of the individual compounds present in extracts from either hybrid line were greater than that of *H. subflexa* (Fisher's LSD, P = 0.05). Similarly, the mean ratios of 16:OH to 16:Ac for the hybrid groups were the same as that of *H. subflexa*. Although four extracts from V line males and six extracts from S line males contained more 16:Ac than 16:OH, the ratios from these individual extracts were not different from the highest ratio of 16:Ac to 16:OH (3.1 to 4.2, 16:Ac to 16:OH) found in an individual extract from *H. subflexa* (χ^2, P = 0.05).

These data indicate that both the blend and amounts of 16:Ac and 16:OH present in hairpencil glands were the same as that present in extracts of *H. subflexa*. Therefore, although the presence and structure of the hairpencil glands are regulated by dominant genes on the Z chromosome of *H. virescens* (Teal and Oostendorp, 1993), the male sex chromosome (Z chromosome) of this species does not exert a dominant effect on pheromone production. Although the data implied that regulation of both the amount and ratio of pheromone components produced by hybrids is controlled by dominant genes in the *H. subflexa* genome, we could not determine if the genes were sex linked or auto-

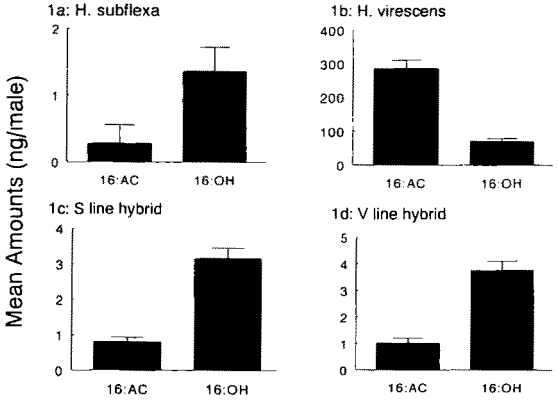

FIG. 1. Comparison of mean amounts (±SE) and ratios of 16:Ac and 16:OH present in extracts obtained from hairpencil glands of males of S line F_1 (N = 125) and V line F_1 hybrids (N = 102) with those found in extracts from males of *H. virescens* and *H. subflexa*.

somal because males of Lepidoptera are homogametic and the hybrid males obtained a Z chromosome from each of the parent species (Table 1).

Backcross 1 Generations. Extracts of males obtained from backcrossing hybrid V line F_1 females to *H. subflexa* (V line female × *H. subflexa* male = VA line) or S line F_1 females to *H. subflexa* (S line female × *H. subflexa* male = SD line) contained the same amounts and ratios of 16:OH and 16:Ac as those obtained from *H. subflexa* males (Figure 2, Table 1). These results were expected because the chromosomal make up of both hybrid lines was either the same as that of the F_1 males or males of *H. subflexa* (Table 1). Although these results confirmed that production of 16:OH and 16:Ac was regulated by dominant genes in the *H. subflexa* genome, they did not indicate if the genes were sex linked.

Extracts obtained from males of backcrosses between V line females and *H. virescens* males (VB line) contained variable amounts of 16:Ac and 16:OH (N = 75) (Figure 2, Table 1). Only six of the extracts contained combined amounts of the two compounds that were greater than 100 ng. These males were considered to be able to produce as much pheromone as males of *H. virescens*. Seven of the extracts contained amounts equivalent to those from *H. subflexa*.

TABLE 1. EFFECT OF HYBRIDIZATION AND BACKCROSSING ON RATIO AND TOTAL AMOUNT OF PHEROMONE IN EXTRACTS OF HAIRPENCIL GLANDS OF MALES[a]

Insect line	Male sex chromosomes[b]	Phenotypes if sex linked, *H. subflexa* dominant	Observed phenotypes for total amount	Observed phenotypes for ratio of 16:Ac and 16:OH
Parent, *H. virescens*	ZvZv	all V type	all V type	all V type
Parent, *H. subflexa*	ZsZs	all S type	all S type	all S type
S line hybrid	ZvZs	all S type	125 S type	125 S type
V line hybrid	ZvZs	all S type	102 S type	102 S type
BC1 SC line	ZvZv	all V type	17 V type[c]	12 V type[c]
			39 I type	34 I type
			6 S type	16 S type
BC1 SD line	ZvZs	all S type	35 S type	35 S type
BC1 VA line	ZsZs	all S type	30 S type	30 S type
BC1 VB line	ZvZs	all S type	6 V type[c]	13 V type[c]
			62 I type	36 I type
			7 S type	26 S type

[a] Phenotype designations are: V = *H. virescens* type, S = *H. subflexa* type, I = intermediate between parental types.

[b] Zv = *H. virescens* Z chromosome, Zs = *H. subflexa* Z chromosome.

[c] There is a difference in these cells between the observed frequency and the frequency expected if the trait were linked to the Z sex chromosome.

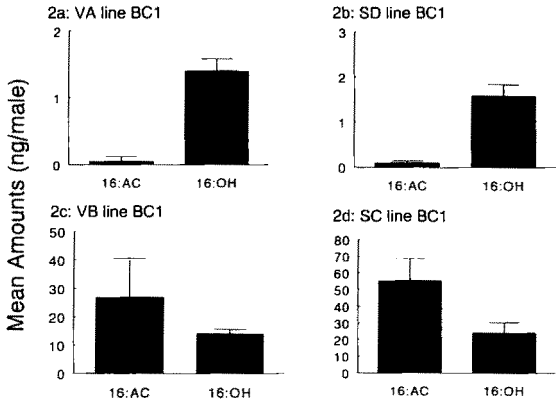

FIG. 2. Mean amounts (±SE) and ratios of 16:Ac and 16:OH present in extracts obtained from hairpencil glands of VA line (N = 30), SD line (N = 35), VB line (N = 75), and SC line (N = 62) backcross 1 generation males.

The remaining 62 extracts contained amounts of these two components that were intermediate between those of *H. subflexa* and *H. virescens*.

Thirteen of the extracts from VB line males contained a 16:Ac to 16:OH ratio similar to that of *H. virescens* (i.e., 4:1) and of these, five contained total amounts greater than 100 ng, while the remaining eight had intermediate amounts of pheromone. Twenty-six extracts contained the same ratio of 16:Ac to 16:OH that was found in *H. subflexa*. The remaining 36 extracts contained ratios of 16:Ac to 16:OH that were intermediate between those of the parent species.

The data obtained from VB line backcross males indicated that genes responsible for the regulation of production of both amounts of 16:Ac and 16:OH and ratios of these compounds were not sex linked. The V line hybrid females obtained their Z chromosome from *H. subflexa*. Thus, backcrossing these females to *H. virescens* males resulted in male progeny that obtained one Z chromosome from *H. virescens* and the other from *H. subflexa* (Table 1). These backcross males had the same sex chromosome makeup as V line hybrid males. If the genes were sex linked, then all of the VB line males should have produced the same amount and ratio of 16:Ac to 16:OH that was produced by males of V line hybrids and *H. subflexa*. However, a significant number of extracts had amounts and ratios of 16:Ac and 16:OH that were intermediate between those of the parent species and others were like *H. virescens*.

Backcrossing S line hybrid females to *H. virescens* (SC line) resulted in males (N = 62) that produced both 16:Ac and 16:OH. However, both the amounts and ratios of the two compounds were variable (Figure 2, Table 1). Seventeen of the extracts contained combined amounts of 16:Ac and 16:OH

equivalent to that of *H. virescens*. Six extracts contained amounts as low as those present in *H. subflexa* extracts, and the remaining 39 contained intermediate amounts of pheromone.

Extracts of 12 SC line males contained the same ratio of 16:Ac to 16:OH as was present in males of *H. virescens* and, of these, seven had as much pheromone as *H. virescens* (Table 1). Another group of 16 extracts contained a ratio of 16:Ac to 16:OH that was like *H. subflexa*. The remaining 34 were intermediate between the two parental ratios.

If the genes regulating the ratio and amount of pheromone were sex linked, then all SC line males should have been identical to *H. virescens* because SC line males obtained both of their Z chromosomes from *H. virescens* (Table 1). However, as occurred with extracts from VB line males, a large number of extracts contained intermediate amounts and ratios of the two components. Additionally other extracts were the same as extracts from males of *H. subflexa*. Therefore, the regulation of both the ratio and amounts of 16:Ac and 16:OH produced by the hairpencil glands of interspecific hybrid and backcross males is controlled by autosomal genes. Additionally, the data from analyses of extracts of both the VB and SC line backcross males show that more than one gene is responsible for regulation of each trait. If single genes were responsible for regulating either trait, then 50% of the populations of males from backcrossing females of either hybrid line to *H. virescens* should have been like this species and the other half should have been like *H. subflexa*. However, approximately 50% of the males from both VB and SC backcross lines were intermediate for both traits (Table 1).

These results, coupled with those from other studies on the regulation of the pheromone-mediated biology of interspecific hybrids between *H. virescens* and *H. subflexa*, indicate that different aspects of the communication system are regulated in different ways. For example, studies on the sex pheromones produced by female hybrids from crosses between *H. subflexa* females and *H. virescens* males showed that these females produced the same blend of pheromone components as do females of *H. virescens* (Klun et al., 1982). This indicates that the regulation of pheromone production by females of this hybrid line appears to be under the dominant regulation of the *H. virescens* genome (Klun et al., 1982). Similarly, both the presence and structure of the male *H. virescens*-type hairpencil glands are dominantly regulated by the *H. virescens* genome (Teal and Oostendorp, 1993). However, in this case, the genes reside on the Z sex chromosome, whereas genes controlling production of the female pheromone are autosomal. The results of this study show that the production of the male hairpencil pheromone in hybrids is under the dominant regulation of autosomal genes of *H. subflexa*. These results, in conjunction with previously published work on *Heliothis*, demonstrate that control of the pheromone communication system in *Heliothis* moths is regulated by a complex genetic system

and support the hypothesis that genes controlling different aspects of the system are not closely linked and are inherited independently in Lepidoptera (Lofstedt et al., 1989).

Acknowledgments—We wish to thank J.A. Meredith and N. Epsky, Insect Attractants Behavior and Basic Biology Research Laboratory, USDA ARS, Gainesville, Florida; R.N. Inserra, Division of Plant Industry, State of Florida, Gainesville, Florida; M.L. Laster, Southern Insect Management Research Laboratory, Stoneville, Massachusetts; and F.I. Proshold, Otis Methods Development Center, Otis ANGB, MA, for helpful reviews of the manuscript.

REFERENCES

BIRCH, M.C., and HEFETZ, A. 1987. Extrusible organs in male moths and their role in courtship behavior. *Bull. Entomol. Soc. Am.* 33:222–229.

CIBRIAN-TOVAR, J., and MITCHELL, E.R. 1991. Courtship behavior of *Heliothis subflexa* (Gn.) (Lepidoptera: Noctuidae) and associated backcross insects obtained from hybridization with *H. virescens* (F.). *Environ. Entomol.* 20:419–426.

HEATH, R.R., MCLAUGHLIN, J.R., PROSHOLD, F., and TEAL, P.E.A. 1991. Periodicity of female sex pheromone titer and release in *Heliothis subflexa* and *H. virescens* (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. Am.* 84:182–189.

KARPENKO, C.P., and PROSHOLD, F.I. 1977. Fertility and mating performance of interspecific crosses between *Heliothis virescens* and *H. subflexa* backcrossed for three generations to *H. subflexa*. *Ann. Entomol. Soc. Am.* 70:737–740.

KLUN, J.A., LEONHARDT, B.A., LOPEZ, J.D., JR., and LACHANCE, L.E. 1982. Female *Heliothis subflexa* (Lepidoptera: Noctuidae) sex pheromone: Chemistry and congeneric comparisons. *Environ. Entomol.* 11:1084–1090.

LASTER, M.L. 1972. Interspecific hybridization of *Heliothis virescens* and *H. subflexa*. *Environ. Entomol.* 1:682–687.

LOFSTEDT, C., HANSSON, B.S., ROELOFS, W., and BENGTSSON, B.O. 1989. No linkage between genes controlling female pheromone production and male response in the European corn borer, *Ostrinia nubilalis* Hubner (Lepidoptera: Pyralidae). *Genetics* 123:553–556.

PROSHOLD, F.I., and LACHANCE, L.E. 1974. Analysis of sterility in hybrids from interspecific crosses between *Heliothis virescens* and *H. subflexa*. *Ann. Entomol. Soc. Am.* 67:445–449.

TEAL, P.E.A., and OOSTENDORP, A. 1993. Effect of interspecific hybridization between *Heliothis virescens* and *H. subflexa* (Lepidoptera: Noctuidae) on the presence and structure of hairpencil glands of males. *Ann. Entomol. Soc. Am.* 88:322–326.

TEAL, P.E.A., and TUMLINSON, J.H. 1989. Isolation, identification and biosynthesis of compounds produced by male hairpencil glands of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 15:413–427.

TEAL, P.E.A., MCLAUGHLIN, J.R., and TUMLINSON, J.H. 1981. Analysis of the reproductive behavior of *Heliothis virescens* (F.) under laboratory conditions. *Ann. Entomol. Soc. Am.* 74:324–330.

TEAL, P.E.A., OOSTENDORP, A., and TUMLINSON, J.H. 1993. Induction of pheromone production in females of *Heliothis virescens* (F.) and *H. subflexa* (Gn.) (Lepidoptera: Noctuidae) during the photophase. *Can. Entomol.* 153:355–366.

TINGLE, F.C., MITCHELL, E.R., and BAUMHOVER, A.H. 1978. Sex pheromone specificity in *Heliothis*. *J. Chem. Ecol.* 4:471–479.